

EX-7

Binding of the B Cell Activation Antigen B7 to CD28 Costimulates T Cell Proliferation and Interleukin 2 mRNA Accumulation

By Peter S. Linsley, William Brady, Laura Grosmaire, Alejandro Aruffo, Nitin K. Damle, and Jeffrey A. Ledbetter

From the Oncogen Division, Bristol-Myers-Squibb Pharmaceutical Research Institute, Seattle, Washington 98121

Summary

A successful immune response requires intercellular contact between T and B lymphocytes. We recently showed that CD28, a T cell surface protein that regulates an activation pathway, could mediate intercellular adhesion with activated B cells by interaction with the B7 antigen. Here we show that CD28 is the primary receptor for B7 on activated peripheral blood T cells, that CD28 binds to B7 in the absence of other accessory molecules, and that interaction between CD28 and B7 is costimulatory for T cell activation. To characterize the binding of CD28 to B7, we have produced genetic fusions of the extracellular portions of B7 and CD28, and immunoglobulin (Ig) C γ 1 chains. ¹²⁵I-labeled B7 Ig bound to CD28-transfected Chinese hamster ovary (CHO) cells, and to immobilized CD28 Ig with a $K_d \sim 200$ nM. B7 Ig also inhibited CD28-mediated cellular adhesion. The function of CD28-B7 interactions during T cell activation was investigated with soluble fusion proteins and with B7-transfected CHO cells. Immobilized B7 Ig and B7⁺ CHO cells costimulated T cell proliferation. Stimulation of T cells with B7⁺ CHO cells also specifically increased levels of interleukin 2 transcripts. These results demonstrate that the CD28 signaling pathway could be activated by B7, resulting in increased T cell cytokine production and T cell proliferation. Cellular interactions mediated by B7 and CD28 may represent an important component of the functional interactions between T and B lymphoid cells.

It has long been known that interactions between T and B lymphocytes play a central role in regulating an immune response (1, 2). More recent studies have shown that activation and differentiation of both T_H and B lymphocytes is dependent upon direct intercellular (cognate) interactions between these cell types. While the specificity of T_H cell-B cell interactions is determined by interaction between the TCR/CD3 complex (3-5) and antigen associated with class II MHC molecules on B cells (6), interactions between other (accessory) cell adhesion molecules are also necessary for a full immune response (7-9). Interactions between accessory receptors and their counter-receptors may increase the avidity of cellular interactions (7); control lymphocyte localization and migration (10); and have direct signaling functions (7, 11, 12) during lymphocyte activation. Accessory receptors and their counter-receptors involved in T_H-B cell interactions include (reviewed in reference 7): CD2 and LFA-3, CD4 and class II MHC molecules, LFA-1, and ICAM-1 and ICAM-2.

The T cell homodimer, CD28, a member of the Ig superfamily (13), has been shown in studies using mAbs to have an accessory function during T cell activation (14). Anti-CD28 mAbs have been shown to costimulate T cell proliferation

induced by a number of polyclonal stimuli (reviewed in reference 14). These mAbs also inhibited alloantigen and soluble antigen-specific T cell responses (15, 16), indicating that CD28-mediated signalling may be crucial during these responses. CD28-mediated T cell activation, unlike that initiated via TCR, was resistant to inhibition by the immunosuppressive agent, cyclosporine (17). Some of the effects of anti-CD28 mAbs appear to result, in part, from the coordinate stimulation of several T cell-derived cytokines (18) through stabilization of their mRNAs (19).

We recently reported that Chinese hamster ovary (CHO)¹ cells transfected with CD28 mediated specific adhesion with certain activated normal and malignant B cells (20). CD28-mediated adhesion was blocked by the mAb BB-1 (21), which recognizes the B cell activation antigen, termed B7, another member of the Ig superfamily (22). COS cells transfected with a cDNA clone encoding B7 adhered specifically to CD28⁺ CHO cells, thereby indicating that the B7 antigen is a counter-receptor for CD28.

¹Abbreviations used in this paper: CHO, Chinese hamster ovary; dhfr, dihydrofolate reductase.

The recognition of CD28 by B7 is a novel mechanism to regulate interaction of T and B lymphocytes. In this paper, we have further characterized biochemical and functional aspects of the interaction between these molecules. Fusion proteins of B7 and CD28 with human Ig C γ 1 chains were expressed and used to measure the specificity and apparent affinity of their interaction. We have also used purified B7Ig fusion protein, as well as CHO cells transfected with B7 to investigate the functional importance of this interaction on T cell activation and cytokine production.

Materials and Methods

Plasmid Construction. Expression plasmids containing cDNAs encoding CD28 (13), CD5 (23), and B7 (22) have been described previously. For initial attempts at expression of soluble forms of CD28 and B7, constructs were made (OMCD28 and OMB7) in which stop codons were introduced upstream of the transmembrane domains and the native signal peptides were replaced with the signal peptide from oncostatin M (24). These were made using synthetic oligonucleotides for reconstruction (OMCD28) or as primers for PCR (OMB7). CD28 Ig and B7 Ig fusion constructs were made in two parts. The 5' portions were made using OMCD28 and OMB7 as templates and the oligonucleotide, CTAGCCACTGAAGCTTCACCATGGGTGTAAGTCTACAC, (corresponding to the oncostatin M signal peptide) as a forward primer, and either TGGCATGGGCTCCTGATCAGGCTTAGAAGGTCCGGGAAA or, TTTGGGCTCCTGATCAGGAAAATGCTCTTGCTTGGT-TGT as reverse primers, respectively. Products of the PCR reactions were cleaved with restriction endonucleases (HindIII and BclI) at sites introduced in the PCR primers and gel purified.

The 3' portion of the fusion constructs corresponding to human Ig C γ 1 sequences was made by a coupled reverse transcriptase (from Avian myeloblastosis virus; Life Sciences Inc., St. Petersburg, FL)-PCR reaction using RNA from a myeloma cell line producing human-mouse chimeric mAb L6 (P. Fell and M. Gayle, unpublished results) as template. The oligonucleotide, AAGCAAGAGCATTTTCTCTGATCAGGAGCCCCAAATCTTCTGACAAAAC-TACACATCCCCACCGTCCCCAGCACCTGAAGTCTCTG, was used as forward primer, and CTTCGACCACTCTAGAA-GCATCCTCGTGCGACCGCGAGAGC as reverse primer. Reaction products were cleaved with BclI and XbaI and gel purified. Final constructs were assembled by ligating HindIII/BclI cleaved fragments containing CD28 or B7 sequences together with BclI/XbaI cleaved fragment containing Ig C γ 1 sequences into HindIII/XbaI cleaved CDM8. Ligation products were transformed into MC1061/p3 cells and colonies were screened for the appropriate plasmids. Sequences of the resulting constructs were confirmed by DNA sequencing (see Fig. 1). CD5 Ig was constructed in identical fashion, using CATTGCACAGTCAAGCTTCCATGCCCCATG-GGTTCTCTGGCCACCTTG, as forward primer and ATCCAC-AGTGCAGTGATCATTTGGATCCTGGCATGTGAC as reverse primer. The PCR product was restriction endonuclease digested and ligated with the Ig C γ 1 fragment as described above. The resulting construct (CD5 Ig) encodes a mature protein comprising residues 1-347 of CD5, two amino acids introduced by the construction procedure (amino acids DQ), followed by the Ig C γ 1 hinge region as shown in Fig. 1.

PCR reactions (0.1 ml final volume) were run in *Taq* polymerase buffer (Stratagene, Torrey Pines, CA), containing 20 μ mol each dNTP; 50-100 pmol of the indicated primers; template (1 ng plasmid or cDNA synthesized from ≤ 1 μ g total RNA using random hex-

amer primer); and *Taq* polymerase (Stratagene). Reactions were run on a thermocycler (Perkin Elmer Corp., Norwalk, CT) for 16-30 cycles (a typical cycle consisted of steps of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C).

Immunostaining and FACS Analysis. Transfected CHO cells or activated T cells were analyzed by indirect immunostaining. Before staining, CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with murine mAbs 9.3 (25) or BB-1 (21), or with Ig fusion proteins (all at 10 μ g/ml in DMEM containing 10% FCS) for 1-2 h at 4°C. Cells were then washed, and incubated for an additional 0.5-2 h at 4°C with a FITC-conjugated second step reagent (goat anti-mouse Ig serum for murine mAbs, or goat anti-human Ig C γ serum for fusion proteins; Tago, Inc., Burlingame, CA). Fluorescence was analyzed on a FACS IV[®] cell sorter (Becton Dickinson and Co., Mountain View, CA) equipped with a four decade logarithmic amplifier.

Cell Culture and Transfections. Stable transfectants expressing CD28, CD5, or B7 were isolated following cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr⁻ CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr (20). Transfectants were then grown in increasing concentrations of methotrexate to a final level of 1 μ M. Lines expressing high levels of CD28 (CD28⁺ CHO) or B7 (B7⁺ CHO) were isolated by multiple rounds of fluorescence-activated cell sorting following indirect immunostaining with mAbs 9.3 or BB-1. Amplified CHO cells negative for surface expression of CD28 or B7 (dhfr⁺ CHO) were also isolated by fluorescence-activated cell sorting from CD28-transfected populations.

COS cells were transfected with expression plasmids using a modification of the protocol of Seed and Aruffo (26). Cells were seeded at 10⁶ per 10 cm diameter culture dish 18-24 h before transfection. Plasmid DNA was added (~ 15 μ g/dish) in a volume of 5 ml of serum-free DMEM containing 0.1 mM chloroquine and 600 μ g/ml DEAE Dextran, and cells were incubated for 3-3.5 h at 37°C. Transfected cells were then briefly treated (~ 2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16-24 h in DMEM containing 10% FCS. At ~ 24 h after transfection, culture medium was removed and replaced with serum-free DMEM (6 ml/dish). Incubation was continued for 3 d at 37°C, at which time the spent medium was collected and fresh serum-free medium was added. After an additional 3 d at 37°C, the spent medium was again collected and cells were discarded.

Purification of Ig Fusion Proteins. The first and second collections of spent serum-free culture media from transfected COS cells were used as source for the purification of Ig fusion proteins. After removal of cellular debris by low speed centrifugation, medium was applied to a column (~ 200 -400 ml medium/ml packed bed volume) of immobilized protein A (Repligen Corp., Cambridge, MA) equilibrated with 0.05 M sodium citrate, pH 8.0. After application of the medium, the column was washed with 1 M potassium phosphate, pH 8, and bound protein was eluted with 0.05 M sodium citrate, pH 3. Fractions were collected and immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8. Fractions containing the peak of A₂₈₀ absorbing material were pooled and dialyzed against PBS before use. Extinction coefficients of 2.4 and 2.8 ml/mg for CD28 Ig and B7 Ig, respectively, by amino acid analysis of solutions of known absorbance. The recovery of purified CD28 Ig and B7 Ig binding activities were nearly quantitative as judged by FACS[®] analysis after indirect fluorescent staining of B7⁺ and CD28⁺ CHO cells.

Cell Separation and Stimulation. PBL were isolated by centrifu-

gation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) and cultured in 96-well, flat-bottomed plates (4×10^4 cells/well, in a volume of 0.2 ml) in RPMI containing 10% FCS. Cellular proliferation of quadruplicate cultures was measured by uptake of [3 H]thymidine during the last 5 h of a 3-d culture. PHA-activated T cells were prepared by culturing PBL with 1 μ g/ml PHA (Wellcome, Charlotte, NC) for 5 d, and 1 d in medium lacking PHA. Viable cells were collected by sedimentation through Lymphocyte Separation Medium before use.

Cell Surface Iodination and Immunoprecipitation. PHA-activated T cells were cell-surface labeled with 125 I using lactoperoxidase and H_2O_2 (27). Nonionic detergent extracts of labeled cells were prepared and subjected to immunoprecipitation analysis as described previously (28).

Radiolabeling of B7 Ig. Purified B7 Ig (25 μ g) in a volume of 0.25 ml of 0.12 M sodium phosphate, pH 6.8 was iodinated using 2 mCi 125 I and 10 μ g of chloramine T. After 5 min at 23°C, the reaction was stopped by the addition of 20 μ g sodium metabisulfite, followed by 3 mg of KI and 1 mg of BSA. Iodinated protein was separated from unreacted 125 I by chromatography on a 5-ml column of Sephadex G-10 equilibrated with PBS containing 10% FCS. Peak fractions were collected and pooled. The specific activity of 125 I-B7 Ig labeled in this fashion was 1.5×10^6 cpm/pmol (2.8×10^7 cpm/ μ g).

B7 Ig was also metabolically labeled with [35 S]methionine. COS cells were transfected with a plasmid encoding B7 Ig as described above. At 24 h after transfection, [35 S]methionine (>800 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to concentrations of 115 μ Ci/ml in DMEM containing 10% FCS and 10% normal levels of methionine. After incubation at 37°C for 3 d, medium was collected and used for purification of B7 Ig as described above. Concentrations of [35 S]methionine-labeled B7 Ig were estimated by comparison of staining intensity after SDS-PAGE with intensities of known amounts of unlabeled B7 Ig. The specific activity of [35 S]methionine-labeled B7 Ig was $\sim 2 \times 10^6$ cpm/ μ g.

Binding Assays. For assays using immobilized CD28Ig, 96-well plastic dishes were coated for 16–24 h with a solution containing CD28Ig (0.5 μ g in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were then blocked with binding buffer (DMEM containing 50 mM BES, pH 6.8, 0.1% BSA, and 10% FCS) (Sigma Chemical Co., St. Louis, MO) before addition of a solution (0.09 ml) containing 125 I-B7 Ig ($\sim 3 \times 10^6$ cpm) or [35 S]-B7 Ig (1.5×10^5 cpm) in the presence or absence of competitor. After incubation for 2–3 h at 23°C, wells were washed once with binding buffer, and four times with PBS. Bound radioactivity was then solubilized by addition of 0.5 N NaOH, and quantified by liquid scintillation or γ counting. When binding of 125 I-B7 to CD28 $^+$ CHO cells was measured, cells were seeded (2.5×10^4 /well) in 96-well plates 16–24 h before the start of the experiment. Binding was otherwise measured as described above.

SDS Page. SDS-PAGE was performed on linear acrylamide gradient gels with stacking gels of 5% acrylamide. Gels were stained with Coomassie Blue, destained, and photographed or dried and exposed to X-ray film (Kodak XAR-5; Eastman Kodak Co., Rochester, NY).

RNA Blot Analysis. RNA was prepared from stimulated PHA blasts by a published procedure (29). Aliquots of RNA (20 μ g) were fractionated on formaldehyde agarose gels and then transferred to nitrocellulose by capillary action. RNA was crosslinked to the membrane by UV light in a Stratalinker (Stratagene), and the blot was prehybridized and hybridized with a 32 P-labeled probe for human IL-2 (prepared from a ~ 600 -bp cDNA fragment provided by Dr. S. Gillis; Immunex Corp., Seattle, WA). Equal loading of

RNA samples was verified both by rRNA staining and by hybridization with a rat glyceraldehyde-6-phosphate dehydrogenase probe (GAPDH, a ~ 1.2 -kb cDNA fragment provided by Dr. A. Purchio, Oncogen).

Results

Construction and Expression of B7 and CD28 Immunoglobulin C γ Fusion Proteins. In initial attempts to make soluble derivative of B7 and CD28, we made cDNA constructs encoding molecules truncated at the NH $_2$ -terminal side of their transmembrane domains. In both cases, the native signal peptides were replaced with the signal peptide from oncostatin M (24), which mediates efficient release of secreted proteins in transient expression assays (J. Kallestad, P. S. Linsley, and W. Brady, unpublished observations). These cDNAs were cloned into an expression vector, transfected into COS cells, and spent culture medium was tested for secreted forms of B7 and CD28. In this fashion, we produced several soluble forms of B7, but in repeated attempts, we were unable to detect soluble CD28 molecules.

We then took the approach of making receptor Ig C γ fusion proteins. Other investigators (23, 30) have shown these molecules to be efficiently produced in transient expression systems, and easily purified and detected using standard immunochemical techniques. The B7 and CD28 extracellular regions, preceded by the signal peptide to oncostatin M, were fused in frame to an Ig C γ 1 cDNA, as shown in Fig. 1 A. During construction, the Ig hinge disulfides were mutated to serine residues to abolish intrachain disulfide bonding. The resulting fusion proteins were produced in COS cells and purified by affinity chromatography on immobilized protein A as described in Materials and Methods. Yields of purified protein were typically 1.5–4.5 mg/liter of spent culture medium.

As shown in Fig. 1 B, the B7 Ig fusion protein migrated during SDS-PAGE under nonreducing conditions predominantly as a single species of M_r 70,000, with a small amount of material migrating as a M_r $\sim 150,000$ species. After reduction, a single M_r $\sim 75,000$ species was observed. The nature of the M_r $\sim 150,000$ species was not investigated further. CD28 Ig migrated as a M_r $\sim 140,000$ species under nonreducing conditions and a M_r $\sim 70,000$ species after reduction, indicating that it was expressed as a homodimer. Since the Ig C γ 1 hinge cysteines had been mutated, disulfide linkage probably involved cysteine residues which naturally form interchain bonds in the CD28 homodimer (25).

Binding Activities of B7 and CD28 Immunoglobulin C γ Fusion Proteins. To investigate the functional activities of B7 Ig and CD28 Ig, we first tested binding to CHO cell lines expressing CD28 or B7. In early experiments, spent culture media from transfected COS cells was used as a source of fusion protein, while in later experiments, purified proteins were used (Fig. 2). Binding was detected by addition of FITC-conjugated goat anti-human Ig second step reagent. B7 Ig was bound by CD28 $^+$ CHO, while CD28 Ig was bound by B7 $^+$ CHO. B7 Ig also bound weakly to B7 $^+$ CHO (Fig. 2), suggesting that this molecule has a tendency to form

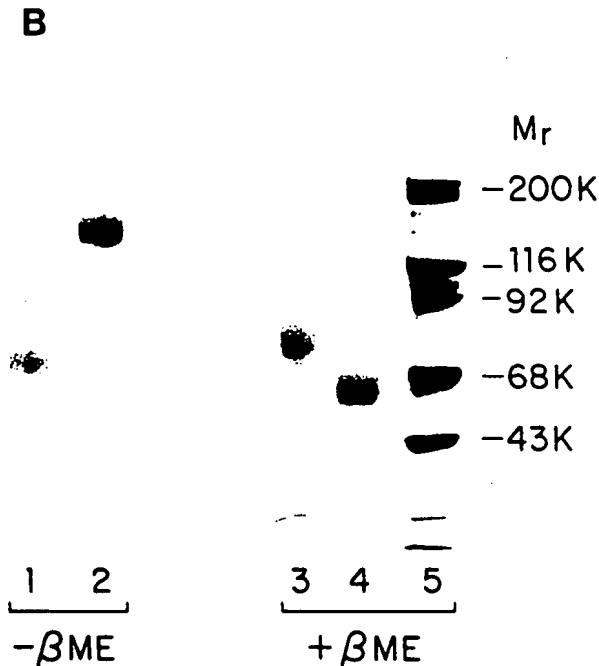
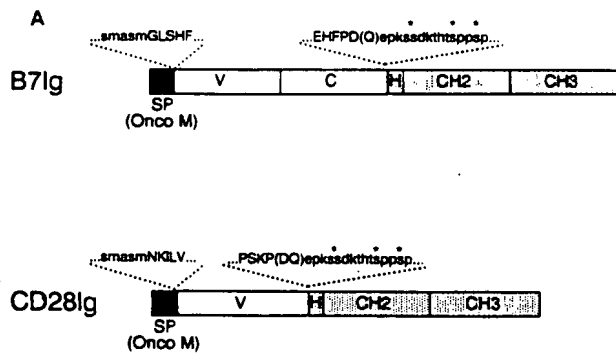


Figure 1. Construction and expression of B7 and CD28 Ig C γ fusion proteins. (A) Maps of B7 Ig and CD28 Ig constructs. cDNA constructs encoding the indicated portions of oncostatin M (dark shaded regions), B7 and CD28 (unshaded regions), and human Ig C γ 1 (stippled regions) were constructed as described in Materials and Methods. Sequences displayed show the junctions between B7 and CD28 (capital letters), and the signal peptide (SP) of oncostatin M at their NH₂ termini, and the hinge (H) of Ig C γ 1 at their COOH termini. Amino acids introduced during construction are indicated in parentheses. Asterisks denote cysteine to serine mutations introduced in the hinge. Boxes labeled V and C denote Ig superfamily-like domains present in CD28 (13) and B7 (22). The CH2 and CH3 domains of Ig C γ 1 are also indicated. (B) Purification of B7 Ig and CD28 Ig. Expression plasmid constructs encoding the fusion proteins displayed above were transfected into COS cells. Serum-free conditioned medium was collected, and Ig C γ -containing proteins were purified by protein A affinity chromatography as described in Materials and Methods. Aliquots (1 μ g) of B7 Ig (lanes 1 and 3) or CD28 Ig (lanes 2 and 4) were subjected to SDS-PAGE (4–12% acrylamide gradient) under nonreducing (- β ME, lanes 1 and 2) or reducing (+ β ME lanes 3 and 4) conditions. Lane 5 shows mol wt markers. Proteins were visualized by staining with Coomassie Brilliant Blue.

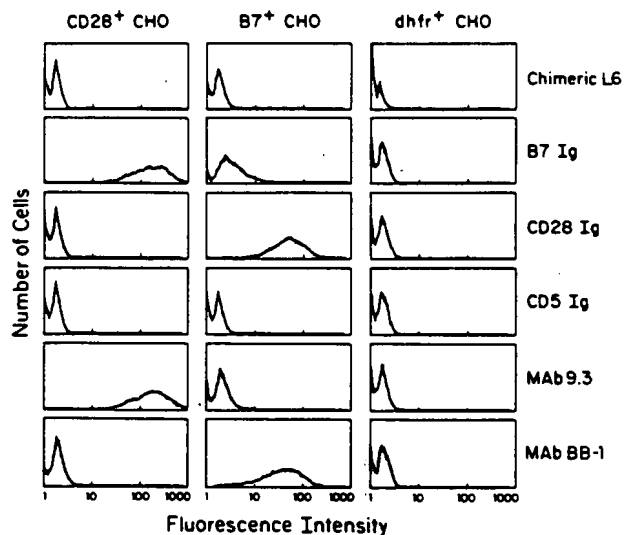


Figure 2. B7 Ig and CD28 Ig bind to transfected CHO Cells. Amplified transfected CHO cells expressing CD28, B7, or dhfr only (no surface marker) were first stained with human Ig C γ 1-containing proteins (chimeric mAb L6, CD28 Ig, B7 Ig, or CD5 Ig), or mouse mAbs (9.3 or BB-1 at 10 μ g/ml), followed by FITC-conjugated anti-human or mouse Ig second step reagents. A total of 10,000 stained cells was then analyzed by FACS[®].

homophilic interactions. No binding was detected of a chimeric mAb containing human Ig C γ 1, or of another fusion protein, CD5 Ig. Thus, B7 Ig and CD28 Ig retain binding activity for their respective counter-receptors.

We next determined the apparent affinity of interaction between B7 and CD28. B7 Ig was either metabolically labeled with [³⁵S]methionine, or iodinated, and radiolabeled derivatives were tested for binding to immobilized CD28 Ig or to CD28⁺ CHO cells. A competition binding experiment using ¹²⁵I-B7 Ig and immobilized CD28 Ig is shown in Fig. 3 A. Binding of ¹²⁵I-B7 Ig was competed in dose-dependent fashion by unlabeled B7 Ig, and by mAbs 9.3 and BB-1. mAb 9.3 was the most effective competitor (half-maximal inhibition at 4.3 nM), followed by mAb BB-1 (half-maximal inhibition at 140 nM) and B7 Ig (half-maximal inhibition at 280 nM). Thus, mAb 9.3 was ~65-fold more effective as a competitor than B7 Ig, indicating that the mAb has greater apparent affinity for CD28. The same relative difference in avidities was seen when [³⁵S]methionine-labeled B7 Ig was used. Chimeric mAb L6 did not significantly inhibit binding; the inhibition at high concentrations in Fig. 3 A was not seen in other experiments. When the competition data were plotted in the Scatchard representation (Fig. 3 B), a single class of binding sites was observed (K_d ~200 nM). An identical K_d was detected for binding of ¹²⁵I-B7 Ig to CD28⁺ CHO cells. Thus, both membrane bound CD28 and immobilized CD28 Ig showed similar apparent affinities for ¹²⁵I-B7.

CD28 is the Primary B7-binding Protein on PHA-activated T Cells. Although B7 Ig bound to immobilized CD28 Ig, and to CD28⁺ CHO cells, it was not known whether B7 Ig could bind to CD28 naturally expressed on T cells. This is

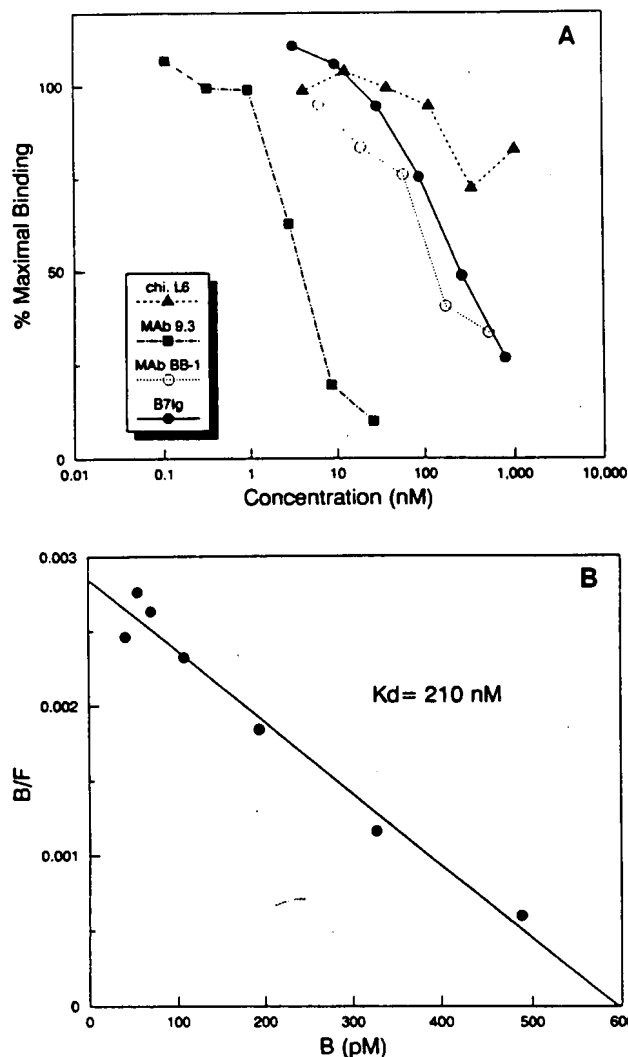


Figure 3. 125 I-labeled B7 Ig binds with high affinity to immobilized CD28 Ig. (A) Competition binding analysis of 125 I-B7 Ig. 96-well plastic dishes were coated with CD28 Ig as described in Materials and Methods. 125 I-labeled B7 Ig (3.3×10^6 cpm, 2×10^6 cpm/pmol) was then added to a concentration of 24 nM in the presence of the indicated concentrations of unlabeled chimeric L6, mAb 9.3, mAb BB-1, or B7 Ig. Plate-bound radioactivity was determined and is expressed as a percentage of radioactivity bound to wells treated without competitor (7,800 cpm). Each point represents the mean of duplicate determinations; replicates generally varied from the mean by $\leq 20\%$. Concentrations were calculated based on a M_r of 75,000 per binding site for mAbs and 51,000 per binding site for B7 Ig. (B) Scatchard analysis of unlabeled B7 Ig competition binding experiment. Data shown in Fig. 3A was replotted in the Scatchard representation and a binding constant (K_d) was estimated from the slope of the line best fitting the experimental data ($r = -0.985$).

an important consideration since the level of CD28 on transfected cells was ~ 10 -fold higher than that found on PHA-activated T cells (20). We therefore tested PHA-activated T cells for binding of B7 Ig by FACS[®] analysis after indirect immunofluorescent staining. As shown in Fig. 4A, these cells bound significant levels of B7 Ig, and binding was inhibited by mAbs 9.3 and BB-1. We also determined the iden-

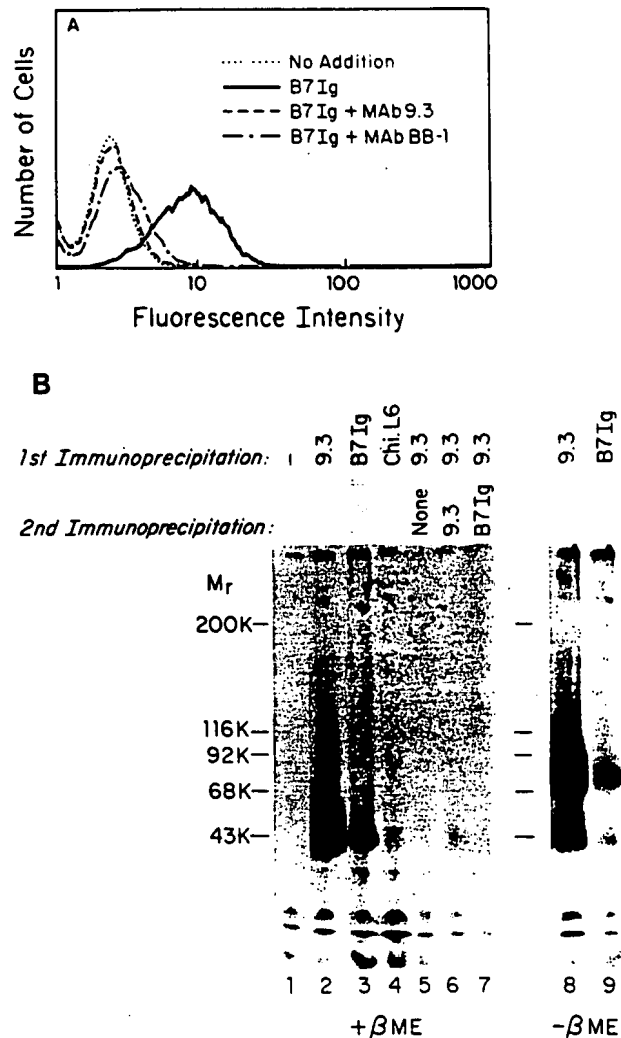


Figure 4. CD28 is the primary B7 Ig binding protein on PHA-activated T cells. (A) FACS[®] profiles of B7 Ig binding to PHA blasts. PHA-stimulated PBL were stained without or with B7 Ig at 10 μ g/ml. Where indicated mAbs 9.3 or BB-1 were added (also at 10 μ g/ml) to cells simultaneously with B7 Ig. Bound mAb was detected with a FITC-conjugated goat anti-human Ig G γ reagent, and stained cells were analyzed by FACS[®]. (B) Autoradiogram of 125 I-labeled proteins immunoprecipitated by B7 Ig. PHA-stimulated PBL were surface labeled with 125 I as described in Materials and Methods. Aliquots of a nonionic detergent extract of labeled cells ($\sim 3 \times 10^8$ cpm in a volume of 0.12 ml) were subjected to immunoprecipitation analysis with no addition (lane 1), mAb 9.3 (5 μ g, lane 2), B7 Ig (10 μ g, lane 3), or chimeric L6 (10 μ g, lane 4). After precipitation of antigen-antibody complexes using formalin-fixed *Staphylococcus aureus*, extracts were then subjected to a second immunoprecipitation analysis with no addition (lane 5), mAb 9.3 (5 μ g, lane 6), or B7 Ig (10 μ g, lane 7). Washed immunoprecipitates were analyzed by SDS-PAGE (5–15% acrylamide gradient) under reducing (+ β -ME, lanes 1–7) or non-reducing conditions (– β -ME, lanes 8 and 9), the gel was dried, and subjected to autoradiography.

tity of B7 Ig-binding proteins by immunoprecipitation analysis of 125 I-surface-labeled cells (Fig. 4B). Both mAb 9.3 and B7 Ig immunoprecipitated a $M_r \sim 45,000$ protein under reducing conditions and $M_r \sim 45,000$ and $\sim 90,000$ proteins

under nonreducing conditions, with the latter form being more prominent. The M_r ~45,000 protein found in the sample precipitated with chimeric mAb L6 was due to spill-over and was not seen in other experiments. mAb 9.3 was more effective at immunoprecipitation than B7 Ig, in agreement with the greater affinity of the mAb (Fig. 3). Identical results were obtained when CD28⁺ CHO cells were used for immunoprecipitation analysis. Preclearing of CD28 by immunoprecipitation with mAb 9.3 also removed B7 Ig-precipitable material, indicating that both mAb 9.3 and B7 Ig bound the same ¹²⁵I-labeled protein. Taken together, the results in this section indicate that CD28 is the major receptor for B7 Ig on PHA-activated T cells.

B7-Binding to CD28 Blocks CD28-mediated Adhesion. mAbs to CD28 have potent biological activities on T cells, suggesting that interaction of CD28 with its natural ligand(s) may also have important functional consequences. As a first step in determining functional consequences of interaction between B7 and CD28, we asked whether B7 Ig could block the CD28-mediated adhesion assay (20). As shown in Fig. 5, B7 Ig blocked CD28-mediated adhesion somewhat less effectively than mAb 9.3 (half-maximal inhibition at 200 nM as compared with 10 nM for mAb 9.3). The relative effectiveness of these molecules at inhibiting CD28-mediated adhesion was similar to their relative binding affinities in competition binding experiments (Fig. 3 A). CD28 Ig failed to inhibit CD28-mediated adhesion at concentrations of up to 950 nM (data not shown), suggesting that much higher levels of CD28 Ig were required to compete with the high local concentrations of CD28 present on transfected cells.

B7 Costimulates T Cell Proliferation. We next investigated whether triggering of CD28 by B7 was costimulatory for

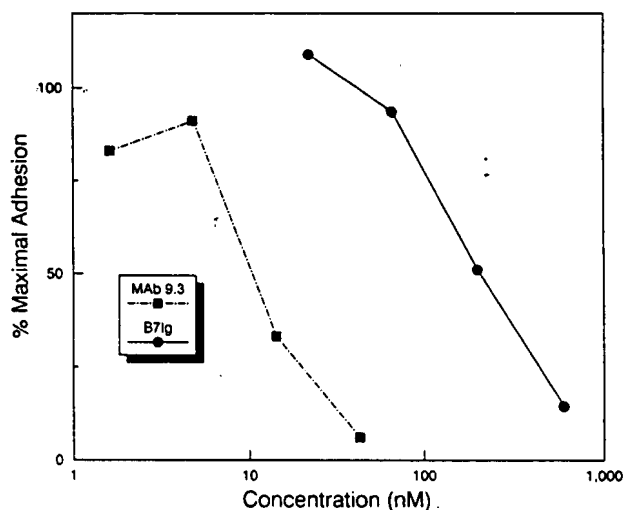


Figure 5. B7 Ig specifically inhibits CD28-mediated adhesion. The adhesion of ⁵¹Cr-labeled PM lymphoblastoid cells to monolayers of CD28⁺ CHO cells was measured as described previously (20) in the presence of the indicated amounts of mAb 9.3 or B7 Ig. Data are expressed as a percentage of cells bound in the absence of competitor (40,000 cpm or ~1.1 × 10⁵ cells). Each point represents the mean of triplicate determinations; coefficients of variation were ≤25%.

Table 1. B7 Ig Is Costimulatory with Anti-CD3 for Proliferation of Peripheral Blood Lymphocytes

| Exp. | CD28 stimulation | ³ H]-T incorporation | |
|------------------------------|-----------------------|---------------------------------|------------|
| | | - Anti-CD3 | + Anti-CD3 |
| <i>cpm × 10⁻³</i> | | | |
| 1 | None | 0.1 | 26.0 |
| | mAb 9.3 (solution) | 0.3 | 156.1 |
| | mAb 9.3 (immobilized) | 0.1 | 137.4 |
| | B7Ig (immobilized) | 0.1 | 174.5 |
| 2 | None | 0.2 | 19.3 |
| | mAb 9.3 (solution) | 0.4 | 75.8 |
| | B7 + CHO cells | 9.4 | 113.9 |
| | dhfr + CHO cells | 23.8 | 22.1 |

PBL were isolated and cultured in the presence of the indicated costimulators of T cell proliferation. Anti-CD3 stimulation was with mAb G19-4 at 1 μg/ml in solution. For CD28 stimulation, mAb 9.3 or B7 Ig were added in solution at 1 μg/ml, or after immobilization on the culture wells by pre-incubation of proteins at 10 μg/ml in PBS for 3 h at 23°C and then washing the culture wells. B7⁺ CHO and control dhfr⁺ CHO cells were irradiated with 1,000 rad before mixing with PBL at a 4:1 ratio of PBL/CHO cells. After culture for 3 d, proliferation was measured by uptake of [³H]thymidine for 5 h. Values shown are means of determinations from quadruplicate cultures (SEM <15%).

T cell proliferation. The ability of B7 Ig to costimulate proliferation of PBL together with anti-CD3 was first explored. In several experiments, B7 Ig in solution at concentrations of 1–10 μg/ml showed only a modest enhancement of prolifer-

Table 2. B7 is Directly Stimulatory for Proliferation of PHA Blasts

| T cells/CHO cells | [³ H]-T incorporation | |
|------------------------------|-----------------------------------|------------------------|
| | + B7 ⁺ CHO | + CD5 ⁺ CHO |
| <i>cpm × 10⁻³</i> | | |
| 25:1 | 92.7 | 15.5 |
| 50:1 | 135.4 | 19.5 |
| 100:1 | 104.8 | 16.8 |
| 200:1 | 90.3 | 17.7 |
| 400:1 | 57.0 | 13.7 |
| 800:1 | 42.3 | 17.6 |

PHA blasts were cultured at 50,000 cells/well with varying amounts of irradiated CHO cell transfectants. After 2 d of culture, proliferation was measured by a 5-h pulse of [³H]thymidine. Shown are means of quadruplicate determinations (SEM <15%). Background proliferation of PHA blasts without added CHO cells was 11,200 cpm. [³H]Thymidine incorporation by irradiated B7⁺ CHO and CD5⁺ CHO cells alone was <1,800 cpm at each cell concentration and has been subtracted from the values shown.

Table 3. Stimulation of Proliferation of PHA Blasts by B7⁺ CHO Cells Is Inhibited by CD28- or B7- specific mAbs

| Stimulation | mAb | [³ H]-T incorporation |
|----------------------|---------|-----------------------------------|
| | | cpm × 10 ⁻³ |
| None | None | 10.8 |
| B7 ⁺ CHO | None | 180 |
| B7 ⁺ CHO | 9.3 Fab | 132 |
| B7 ⁺ CHO | BB-1 | 98.3 |
| B7 ⁺ CHO | LB-1 | 196 |
| CD7 ⁺ CHO | None | 11.5 |
| CD7 ⁺ CHO | 9.3 Fab | 10.0 |
| CD7 ⁺ CHO | BB-1 | 10.0 |
| CD7 ⁺ CHO | LB-1 | 11.3 |

PHA blasts were stimulated as described in Table 2 with irradiated CHO cells at a ratio of 100:1 T cells/CHO cells. mAbs were added at 10 µg/ml at the beginning of culture. mAb LB-1 (21) is an isotype-matched control for mAb BB-1. Proliferation was measured by uptake of [³H]-thymidine during a 5-h pulse after 2 d of culture. Values represent means of quadruplicate cultures (SEM <15%).

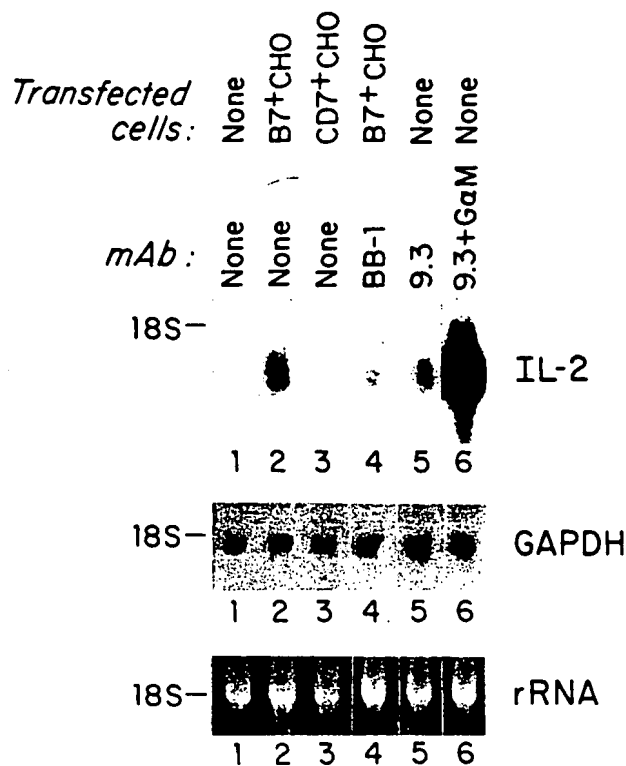


Figure 6. B7 stimulates accumulation of IL-2 mRNA. PHA blasts (5×10^7) were mixed with transfected CHO cells (at a ratio of 40:1 T cells/CHO cells), and/or mAbs as indicated. mAb 9.3 was used at 10 µg/ml. mAb BB-1 (20 µg/ml) was added 1 h before addition of B7⁺ CHO cells. When mAb 9.3 was crosslinked, goat anti-mouse Ig (40 µg/ml) was added 10 min after addition of mAb 9.3. Cells were incubated for 6 h at 37°C. RNA was isolated and subjected to RNA blot analysis, using ³²P-labeled IL-2 or GAPDH probes.

ation even though the anti-CD28 mAb 9.3 was effective. Because CD28 crosslinking has been identified as an important determinant of CD28 signal transduction (31), B7 Ig was also compared to 9.3 when immobilized on plastic wells (Table 1, Exp. 1). Under these conditions, B7 Ig was able to enhance proliferation and compared favorably with mAb 9.3. B7⁺ CHO cells also were tested and compared with control dhfr⁺ CHO cells for costimulatory activity on resting lymphocytes (Table 1, Exp. 2). In this experiment, proliferation was seen with dhfr⁺ CHO cells in the absence of anti-CD3 mAb because of residual incorporation of [³H]thymidine after irradiation of these cells (data not shown). This stimulation was not enhanced by anti-CD3 mAb and was not seen in other experiments (Tables 2 and 3) where transfected CHO cells were added at lower ratios. However, B7⁺ CHO cells were very effective at costimulation with anti-CD3 mAb, indicating that cell surface B7 had similar activity in this assay as anti-CD28 mAbs.

We also tested whether B7⁺ CHO cells could directly stimulate proliferation of resting PHA blasts which respond directly to CD28 crosslinking by mAb 9.3. Again, the B7⁺ CHO cells were very potent in stimulating proliferation (Table 2) and were able to do so at very low cell numbers (PHA blast:B7⁺ CHO ratios of >800:1). The control CD5⁺ CHO cells did not possess a similar activity. (In a number of different experiments neither dhfr⁺ CHO, CD5⁺ CHO, nor CD7⁺ CHO cells stimulated T cell proliferation. These were therefore used interchangeably as negative controls for effects induced by B7⁺ CHO cells). The stimulatory activity of B7⁺ CHO was further shown to result from CD28/B7 interaction, since mAb BB1 inhibited stimulation by the B7⁺ CHO cells without affecting background proliferation in the presence of CD7⁺ CHO cells (Table 3). mAb LB-1 (21), an IgM mAb to a different B cell antigen, did not inhibit proliferation. mAb 9.3 (Fab fragments) inhibited proliferation induced by B7⁺ CHO but not background proliferation seen with CD7⁺ CHO cells. The experiments show that B7 is able to stimulate signal transduction and augment T cell activity by binding to CD28, but that crosslinking is required and B7 expressed on the cell surface is most effective.

B7 Stimulates IL-2 mRNA Accumulation. We also investigated effects of CD28/B7 interactions on IL-2 production by analyzing transcript levels in PHA-blasts stimulated with B7⁺ CHO cells or CD7⁺ CHO cells. RNA was prepared from stimulated cells and tested by RNA blot analysis for the presence of IL-2 transcripts (Fig. 6). B7⁺ CHO cells, but not CD7⁺ CHO cells, induced accumulation of IL-2 transcripts. Induction by B7⁺ CHO cells was partially blocked by mAb BB-1. Induction by B7⁺ CHO cells was slightly better than achieved by mAb 9.3 in solution, but less effective than mAb 9.3 after crosslinking with goat anti-mouse Ig. Thus, triggering of CD28 by cell surface B7 on apposing cells stimulated IL-2 mRNA accumulation.

Discussion

We have used soluble Ig Cγ fusions of both CD28 and B7 to measure the strength of their interaction. The apparent

K_d value for this interaction (~ 200 nM) is within the range of affinities observed for mAbs (2–10,000 nM; reference 32) and compares favorably with the affinities estimated for other lymphoid adhesion molecules. Schneek et al. (33) estimated the affinity ($K_d \sim 100$ nM) between a murine T cell hybridoma TCR and soluble alloantigen (class I MHC molecules). A K_d of 400 nM was measured between CD2 and LFA3 (34). The affinity of CD4 for class II MHC, while not measured directly, was estimated (35) to be $\geq 10,000$ times lower than the affinity of gp120-CD4 interactions ($K_d = 4$ nM; reference 36). Thus, the affinity of B7 for CD28 appears greater than affinities reported for some other lymphoid adhesion systems.

The degree to which the apparent K_d of CD28/B7 interaction reflects their true affinity, as opposed to their avidity, depends on the valency and/or aggregation of the fusion protein preparations. In preliminary experiments, we examined the degree of aggregation of these preparations by size fractionation (TSK G3000SW column eluted with PBS). Under these conditions, B7 Ig eluted at $M_r \sim 350,000$, and CD28 Ig at $M_r \sim 300,000$. Both proteins thus behaved in solution as larger molecules than they appeared by SDS-PAGE (Fig. 1), suggesting that they may form higher aggregates. Alternatively, these results may indicate that both fusion proteins assume extended conformations in solution, resulting in large Stokes radii. Regardless, the interaction we measured using soluble proteins probably underestimates the true avidity between CD28 and B7 in their native membrane-associated state.

The relative contribution of different adhesion systems to the overall strength of T cell–B cell interactions is not easily gauged, but is likely a function of both affinity/avidity and the densities on apposing cell surfaces of the different receptors and counter-receptors involved. Since both CD28 and B7 are found at relatively low levels on resting lymphoid cells (16, 22), they may be less involved than other adhesion systems (7) in initiating intercellular interactions. The primary role of CD28/B7 interactions may be to maintain or amplify a response subsequent to induction of these counter-receptors on their respective cell types.

While occupancy or crosslinking of the T cell antigen receptor is sufficient to initiate a T cell immune response, full activation requires additional costimulatory signals. In some cases, soluble molecules such as IL-1 can provide costimulatory activity, but in other cases the nature of the molecule(s) involved is unclear (8, 9). Schwartz (9) has recently reviewed evidence for a costimulatory activity on antigen-presenting cells which may determine whether TCR occupancy leads to a productive or anergic response in murine T_H clones. This activity appears to require cell-cell contact, leads to increased IL-2 production, and may operate through a different signal-transducing pathway than the TCR (9). All of these characteristics are shared with the CD28 pathway (reviewed in reference 14), leading to the intriguing possibility that CD28 may be one of these functionally defined costimulatory molecules. In agreement with this possibility, is the finding by Ledbetter et al. (31) that anti-CD28 mAb

can partially overcome inhibition of T cell proliferation induced by internalization of the TCR. Studies of the involvement of CD28/B7 interactions in various experimental systems will be required to fully evaluate the role of these costimulatory interactions.

Binding of B7 to CD28 on T cells was costimulatory for T cell proliferation (Tables 1–3), suggesting that some of the biological effects of anti-CD28 mAbs result from their ability to mimic T cell activation resulting from natural interaction between CD28 and its counter-receptor, B7. mAb 9.3 has greater affinity for CD28 than does B7 Ig (Figs. 4 and 5), which may account for the extremely potent biological effects of this mAb (14) in costimulating polyclonal T cell responses. Surprisingly, however, anti-CD28 mAbs are inhibitory for antigen-specific T cell responses (15, 16). This may indicate that antigen-specific T cell responses are dependent upon costimulation via CD28/B7 interactions, and that inhibition therefore results from blocking of CD28 stimulation. Despite the inhibition, CD28 must be bound by mAb under these conditions, implying that triggering by mAb is not always equivalent to triggering by B7. Although mAb 9.3 has higher apparent affinity for CD28 than B7 (Fig. 3), it may be unable under these circumstances to induce the optimal degree of CD28 clustering (31) for stimulation.

CD28/B7 interactions may also be important for B cell activation and/or differentiation. We have recently observed that mAbs 9.3 and BB-1 block T_H cell-induced Ig production by B cells (N. Damle, P. S. Linsley, and J. A. Ledbetter, unpublished results). This blocking effect may be due in part to inhibition by these mAbs of production of T_H -derived B cell-directed cytokines, but may also involve inhibition of B cell activation by interfering with direct signal transduction via B7. These results suggest that cognate activation of B lymphocytes, as well as T_H lymphocytes, is dependent upon interaction between CD28 and B7.

The mechanism of IL-2 transcript induction in T cells by B7⁺ CHO cells (Fig. 6) is not currently known. It is well known that mRNAs for cytokines and other inflammatory mediators are characterized by the presence in their 3' untranslated regions of AU-rich motifs (37), which control mRNA stability (38). Studies by Lindsten et al. (19) showed that anti-CD28 mAbs stimulated production of several T cell-derived cytokines by stabilizing transcripts for these molecules. However, as discussed by June et al. (14), anti-CD28 mAbs may also enhance transcription of IL-2 mRNA. Whatever the mechanism of transcript accumulation by CD28/B7 interactions, regulation of cytokine production by cell-cell contact has also been shown in other systems (39). Many effector functions of T lymphocytes are cytokine mediated (40), and control points in the synthetic pathways of these molecules are therefore extremely important. As dysfunctional cytokine production has been implicated as a contributing factor in several human diseases (40), it will be important to determine how B7/CD28 interactions regulate T cell cytokine production in various pathological states.

We thank Mark Stebbins for advice on purification of fusion proteins and for analytical HPLC analyses of these proteins, Margit Gayle for help with cDNA synthesis and RNA from the chimeric L6 transfectoma, Virginia Smith for amino acid analyses, Derek Hewgill for help with the FACS, Dr. Steve Kanner for a critical review of the manuscript, and Dr. Karl Erik Hellstrom for continued support and encouragement.

Address correspondence to Peter S. Linsley, Oncogen Division, Bristol-Myers-Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, WA 98121.

Received for publication 20 November 1990 and in revised form 10 December 1990.

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